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14. ABSTRACT

We have previously shown that manipulating lymphocytes through Tim1 receptor, a membrane protein that regulates the vigor and differentiation of T cells, using an agonist monoclonal antibody, enhances cytotoxic lymphocyte (CTL) responses to a model prostate tumor-associated antigen (SV40 T antigen) in tumor-free and tumor-bearing mice. This provided a tool to break immune tolerance to prostate tumor antigens in prostate cancer. We now demonstrate, using Tim1 deficient mice, that the effect of the antibody is mediated through Tim-1. We additionally demonstrate that its effect is maintained in humanized HLA-A2.1 transgenic mice when these mice are immunized with peptides derived from the prostate tumor antigens ERG and SIM2. A similar effect was obtained in HLA-A2.1/ERG and HLA-A2.1/ERG/TRAMP. Our findings provide evidence that manipulating Tim1 with an agonist antibody at time of vaccination strengthens cytotoxic responses to mouse and human prostate tumor-associated antigens in healthy and prostate tumor-bearing mice. This represents a major step towards preclinical testing of this strategy against prostate tumor growth and progression and its translation into therapy for prostate cancer in humans.

15. SUBJECT TERMS
Prostate Cancer; Immune tolerance; Immunotherapy; Tim-1 receptor

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INTRODUCTION:

Much of the recent excitement in the field of cancer immunotherapy has been generated by the observation in clinical trials that robust anti-tumor immune responses can be generated through vaccination, and that inhibitory immune checkpoint receptors, such as CTLA-4, PD-L1 and LAG-3, can be blocked by antibodies with profound effects in mouse models and in patients (1). TIM-1 (T cell Immunoglobulin mucin) receptor is expressed on T cells and DCs. It strictly regulates the vigor and nature of the immune response (2-4) through its interaction with its ligand TIM-4 (5). It has been reported that administration of an anti-TIM-1 antibody to mice at the time of immunization with inactivated influenza provides an adjuvant effect promoting antigen-specific T cell responses(6). Of relevance to cancer immunotherapy are the remarkable effects of TIM-1 stimulation on preformed or induced regulatory T cells. TIM-1 stimulation triggered marked down regulation of Foxp3, GITR, TGF-β, CTLA-4 gene expression and amplified IL-17 gene expression (4, 7). Such T cells are totally devoid of immunoregulatory capacity and would, theoretically, not exhibit the powerful silencing effects that Regulatory T Cells exert on CTLs to impede anti-tumor immune responses.

In the project supported by this award, we proposed to use a strategy that combines prostate cancer vaccines and TIM1 receptor manipulation to generate strong, anti-tumor cytotoxic lymphocyte responses for the eradication of prostate tumors in transgenic mice.

In the first year, we have demonstrated that immunization of mice with a prostate-specific antigen, in the presence of an agonist anti-TIM1 monoclonal antibody, resulted in significant recovery of cytotoxic lymphocyte response that is otherwise lost to peripheral tolerance. The ability of TIM1 manipulation to circumvent immune tolerance to tumor antigens seems to depend on the tumor load, suggesting that this intervention might be more efficient in early stages of cancer or at advance stages in combination of other anti-cancer therapies that aim at reducing tumor load.

BODY:

Task 1-b) Using Tim-1- deficient WT and TRAMP mouse:

This task was deferred to year 2 because the TIM1 KO mouse colony and TRAMP/TIM1 KO hybrid were being generated. The task was intended to use the Tim1 deficient WT and TRAMP mice to demonstrate the specificity of the agonist antibody we are using as a tool to break tolerance to T antigen.

TIM1 KO and TRAMP/TIM1 KO mice were immunized with T antigen producing WT19 cell line intraperitoneally. Four hours later, a single dose of agonist anti-Tim1 antibody or isotype control antibody was administered. T antigen-specific CTL response was monitored by IFN-g ELISPOT in response to Tag IV peptide in vitro using splenocytes from immunized mice.

Our data show no differences between isotype and anti-Tim1-treated mice in both WT and TRAMP mice (**Figure 1**). This is consistent with our findings in the first year of this

award where we have used a combination of agonist and antagonist anti-Tim1 antibodies to demonstrate specificity.

Task 2: To explore the role of manipulating Tim-1 pathway in overcoming human HLA-restricted tolerance in transgenic mouse models expressing human prostate TAA.

Task 2-a) Evaluation of immunogenicity and anti-tumor effects of the ERG and SIM2 peptides:

The CTL response to ERG- and SIM2-derived, HLA-A2.1-restricted peptides was tested in HHD (HLA-A2.1 transgenic) mice and was measurable by ELISPOT in spleens and prostate-draining lymph nodes, although weaker than what we have previously seen with SV40 T antigen or full-length PSA (8-11). Treatment with the agonist anti-TIM1 antibody at time of immunization resulted in a significant enhancement of the response for both peptides (**Figure 2**).

Task 2-b) Impact of developing prostate cancers on ERG- and SIM2-specific CTL tolerance:

As mentioned in last year's progress report, we took the decision to switch from the whole body $PTEN^{+/-}$ mouse to the TRAMP mouse to accomplish this task because of the hyper-activation of T lymphocytes in PTEN haplo-insufficient mice. This hyper-activation is a consequence of reduced suppression of PI3K.

For ERG peptide testing, Pb-ERG mice were crossed to HHD mice to generate the ERG/HHD hybrid. This hybrid was further crossed to TRAMP to generate the ERG/HHD/TRAMP mouse.
For SIM2 peptide testing, HHD/TRAMP mouse was generated. Only 25% of male HHD/TRAMP express SIM2 in prostate tumors as we have shown using RT-PCR, a limitation that will required a large number of mice. These SIM2-related experiments are underway, and testing of tumors for SIM2 expression status will be performed upon sacrifice of mice.

Double and triple hybrid mice were immunized with ERG- or SIM-2-derived, HLA-A2.1-restricted peptides. Splenocytes and prostate draining lymph node lymphocytes were tested for their ability to secrete IFN-γ in response to antigen-specific restimulation in vitro.
Probasin-driven expression of ERG in HHD mice clearly results in a reduced anti-ERG CTL response upon immunization with ERG157, ERG295 and ERG 412 peptides (**Figure 3**). However, a part of loss of response is recovered upon addition of the agonist anti-TIM1 antibody at time of immunization.

To test the affect of anti-TIM1 antibody in the context of a tumor-imposed immune tolerance, we generated the HHD/ERG/TRAMP mice. Generating this triple hybrid mouse takes a lot of time and we have been able to generate only a few animals so far. Immunization of HHD, HHD/ERG and HHD/ERG/TRAMP mice with an ERG-derived

peptide shows that immune tolerance to this antigen is induced by prostatic expression in the absence of tumors, and that prostate tumors bring the level of tolerance even lower, although the response is till detectable (**Figure 4**). More triple hybrid mice are being generated to test the effect of anti-TIM1 antibody on tumor-induced immune tolerance.

KEY RESEARCH ACCOMPLISHMENTS:

- We demonstrated that anti-TIM1 treatment of immunized HHD and ERG/HHD mice leads to overcoming immune tolerance to ERG-derived, HLA-A2.1-restricted epitopes. Immune tolerance was not totally circumvented using this treatment, even with higher doses of antibody.
- We showed treatment of T antigen-immunized Tim1 KO mice and TRAMP/Tim1 KO mice with the agonist anti-TIM1 Ab does not result in enhanced CTL responses to Tag-restimulation in the ELISPOT assay.
- We have been able to generate a few male HHD/ERG/TRAMP mice and their immunization shows that they exhibit a partial immune tolerance to prostatic ERG.

REPORTABLE OUTCOMES:

Articles in Preparation:

Arredouani *et al.* Circumventing immune tolerance to prostate tumor-associated antigens through manipulation of Tim-1 receptor.

Presentations/Abstracts. Data generated by this project were presented by the PI of this award at the following meetings:

1) Poster Presentation: Innovative Minds in Prostate Cancer Today (IMPaCT) conference. March 10, 2011. Orlando, FL.

Targeting Tim-1 to Circumvent Immune Tolerance in Prostate Cancer . Arredouani MS, Ph.D. Yue W, M.S., Dunn L, B.S., Putheti P, Ph.D., Strom TB, M.D., Sanda MG, M.D. Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA

2) Poster Presentation: 4th Annual Multi-Institutional Prostate Cancer Program Retreat. March 21 – 23, 2011. Ft Lauderdale, FL.

Molecular Profiling of T lymphocytes in Prostate Cancer. Arredouani MS, Ph.D., Yue W, M.S., Lu B, Ph.D., Dunn L, B.S., Finke J, Asara J, Ph.D., Sanda MG, M.D.

3) Oral Presentation. Dana-Farber/Harvard Cancer Center, Cancer Immunology Seminar Series.

Insights into the mechanisms of immune tolerance to prostate tumor antigens.

Mice. The following hybrid mice were generated for the purpose of this award:

- Tim1 KO/TRAMP
- Pb-ERG/HHD
- Pb-ERG/HHD/TRAMP
- HHD/TRAMP

CONCLUSION:

In the last 12 months, we have shown the specificity of anti-TIM1 treatment through the use of TIM1 KO and TRAMP/TIM1 KO mice. Unlike C57BL/6 mice, these 2 genetically modified mice do not respond to antibody treatment, confirming the specificity of the antibody and discarding the possibility that it acts through its interaction with TCR/CD3 complex (12).

We have also demonstrated that anti-TIM1 targeting enhances specific CTL responses to ERG- and SIM2-derived, HLA-A2.1-restricted epitopes in humanized mice that exhibit prostate-specific antigen expression. Targeting TIM1 seems to work even in mice that exhibit deep immune tolerance to antigen. This proof brings us one step closer to demonstrating anti-tumor efficacy of a potential peptide/anti-TIM1 vaccine formulation.

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APPENDICES: None

SUPPORTING DATA:

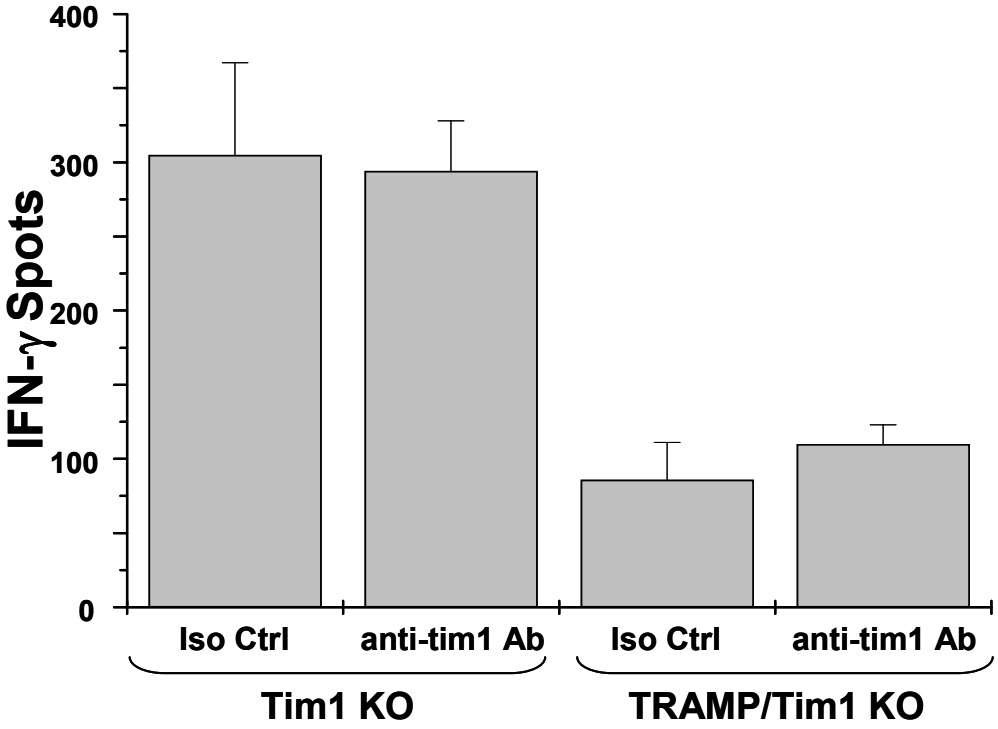

Figure 1: Agonist anti-Tim1 monoclonal antibody exerts its action through TIM-1. Male Tim1 KO and TRAMP/Tim1 KO mice were immunized with the Tag expressing WT19 cell line i.p. and either 200 μg of anti-Tim1 mAb or its isotype control Ab. Tag-specific CTL responses in the spleen were evaluated in response to Tag IV MHC-I-restricted peptide restimulation suing an IFN-γ elispot assay. At least 3 mice were tested per group.

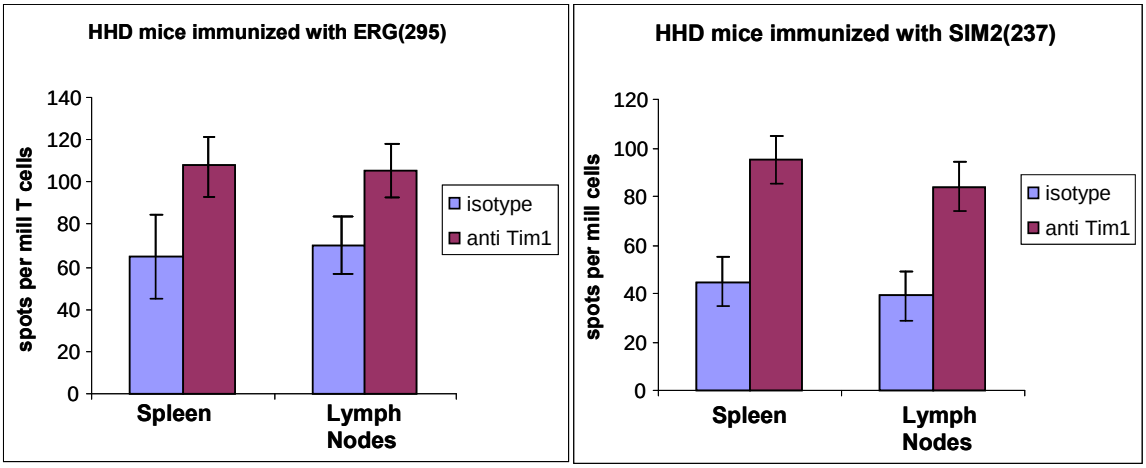

Figure 2: Agonist anti-Tim1 monoclonal antibody enhances CTL responses to human prostate tumor-associated antigens. Male HLA-A2.1 transgenic mice (HHD) were immunized with immunogenic peptides derived from human ERG or SIM2 together with the agonist anti-TIM1 antibody or its isotype control antibody. IFN-γ ELISPOT was performed 10 days post-immunization using splenocytes or prostate-draining lymph node cells.

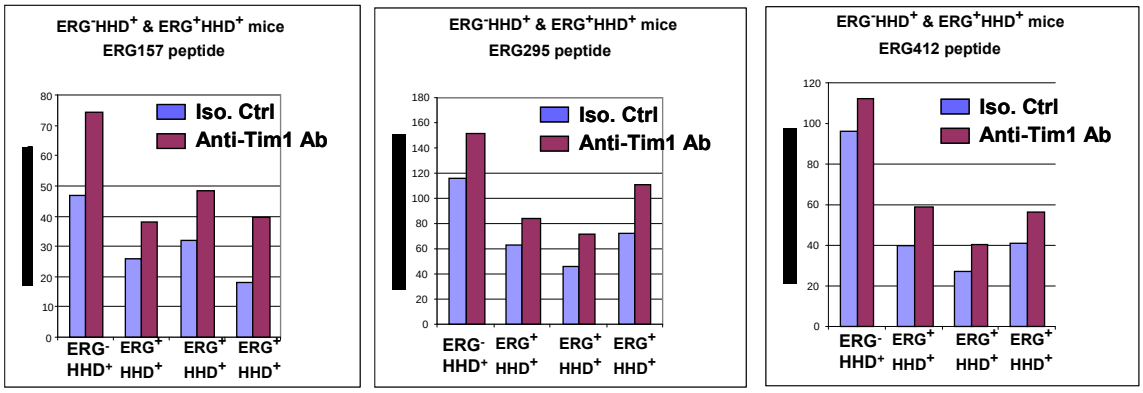

Figure 3: Agonist anti-Tim1 monoclonal antibody partially circumvents immune tolerance to human prostate tumor-associated antigens. Male HLA-A2.1 transgenic mice (HHD, homozygous) were crossed with Pb-ERG (Homozygous) mice. The offspring was either $ERG^{-}HHD^{+}$ & $ERG^{+}HHD^{+}$. Male offspring was immunized with one of the three immunogenic peptides derived from human ERG together with the agonist anti-TIM1 antibody or its isotype control antibody. IFN-γ ELISPOT was performed using splenocytes 10 days post-immunization.

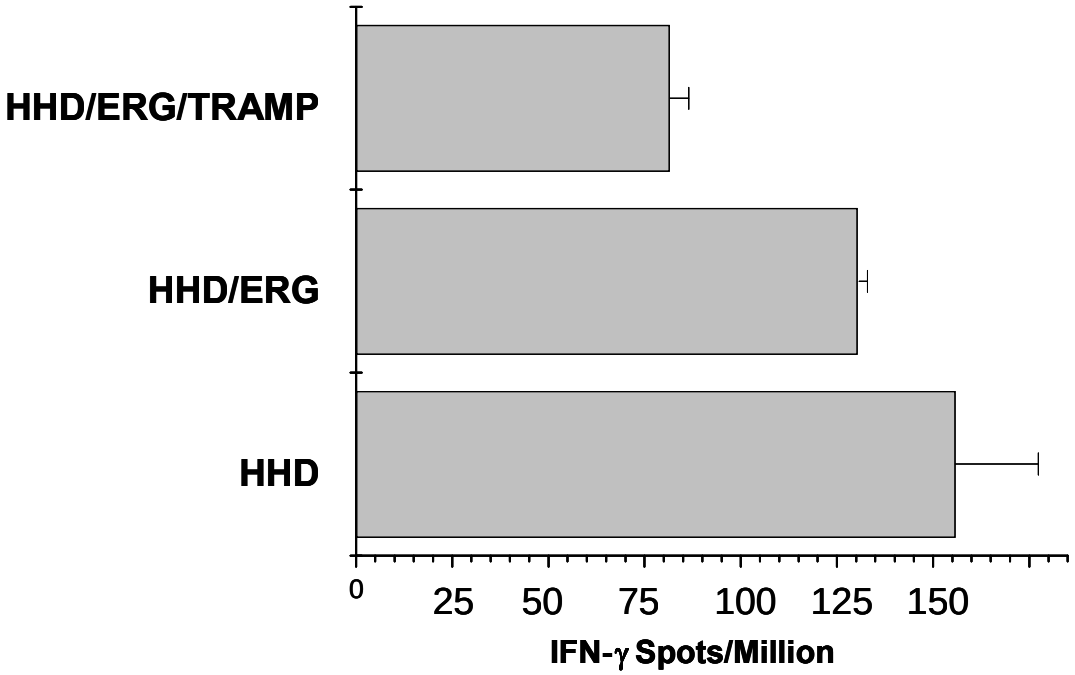

Figure 4: Prostate tumors reduce but do not fully abrogate CTL responses to human TAA. Male HHD, HHD/ERG or HHD/ERG/TRAMP were immunized with ERG295 peptide. IFN-γ ELISPOT was performed 10 days post-immunization using splenocytes.